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Multiple Pathways for Toluene Degradation in *Burkholderia* sp. Strain JS150

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***Burkholderia* (*Pseudomonas*) sp. strain JS150 uses multiple pathways for the metabolism of catechols that result from degradation of aromatic compounds. This suggests that the strain also uses multiple upstream pathways for the initial hydroxylation of aromatic substrates. Two distinct DNA fragments that allowed *Pseudomonas aeruginosa* PAO1c to grow with benzene as a sole carbon source were cloned from strain JS150. One of the recombinant plasmids containing the initial steps for the degradative pathway contained a 14-kb DNA insert and was designated pRO2016. We have previously shown that the DNA insert originated from a plasmid carried by strain JS150 and contained genes encoding a multicomponent toluene-2-monooxygenase (*tbmABCDEF*) as well as the cognate regulatory protein (*tbmR*) that controls expression of the 2-monooxygenase (G. R. Johnson and R. H. Olsen, Appl. Environ. Microbiol. 61:3336–3346, 1995). Subsequently, we have identified an additional region on this DNA fragment that encodes toluene-4-monooxygenase activity. The toluene-4-monooxygenase activity was also regulated by the *tbmR* gene product. A second DNA fragment that allowed *P. aeruginosa* to grow with benzene was obtained as a 20-kb insert on a recombinant plasmid designated pRO2015. The DNA insert contained genes encoding toluene-4-monooxygenase activity but no toluene-2-monooxygenase activity. The pRO2015 insert originated from the chromosome of strain JS150, unlike the region cloned in pRO2016. Southern blots and restriction map comparisons showed that the genes for the individual 4-monooxygenases were distinct from one another. Thus, strain JS150 has been shown to have at least three toluene/benzene monooxygenases to initiate toluene metabolism in addition to the toluene dioxygenase reported previously by others.**

Burkholderia (formerly *Pseudomonas*) sp. strain JS1 and the mutant strains derived from JS1 are unique in their ability to use several pathways for the metabolism of substituted aromatic compounds. This ability is reflected by the number of aromatic compounds that these strains can use as growth substrates (4, 5, 23). For example, *Burkholderia* sp. strain JS150, a nonencapsulated mutant of strain JS1, has been reported to synthesize four ring fission (lower) pathways and three distinct dioxygenases for the initial oxidation of substituted benzenes. The multiplicity of pathways enables strain JS150 to grow with benzene, toluene, ethylbenzene, halogen-substituted benzenes, or naphthalene as sole carbon sources. In addition, there is evidence that strain JS150 is able to simultaneously synthesize enzymes for multiple lower pathways to accommodate differently substituted catechols that result from degradation of complex mixtures of aromatic compounds (4). To our knowledge, similar capabilities have not been described for other naturally occurring bacterial isolates, and this uncommon observation prompted our study of strain JS150. Specifically, we were interested in cloning and characterizing genes encoding degradative pathways for aromatic compounds. This information could provide a genetic basis for the broad range of aromatic compound growth substrates for strain JS150 as well as insight into the mechanisms that the strain uses to adapt to this metabolism.

We have previously reported the characterization of the genes for one degradative pathway from strain JS150, a toluene/benzene-2-monooxygenase (Tb2m) and its cognate regulatory protein. These genes were contained within a 14-kb DNA fragment on the recombinant plasmid designated pRO2016 (12) and are associated with the transformation of several substrates, including phenol, cresols, benzene, toluene, and chlorobenzene. In this report, we describe two additional monooxygenases from strain JS150 which hydroxylate aromatic compounds. On plasmid pRO2016, we have identified a locus encoding a second monooxygenase, toluene/benzene-4-monooxygenase (Tb4m), and its regulation. In addition, we report on a third monooxygenase for substituted benzene degradation from strain JS150. The genes for these functionally redundant, monooxygenase-initiated pathways appear to be carried together with those for the dioxygenase-initiated pathways from strain JS150 (4, 20). Preliminary reports on this work have been presented previously (11, 13).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *Burkholderia* sp. strain JS150 was routinely grown on complex plate count medium (TNA) (6) or Vogel-Bonner mineral salts medium (VB) (25) with 0.2% lactate. *Pseudomonas aeruginosa* PAO1c (8) or *P. aeruginosa* PAO4032 (*catA*) (9) carrying pRO1727 (2) or pRO1614 (18) and their derivatives were grown on TNA with carbenicillin or ticarcillin (500 or 250 μg/ml, respectively). *P. aeruginosa* strains carrying recombinant derivatives of pRO2321 (27) were grown on VB with trimethoprim (600 μg/ml). A minimal basal salts medium (BM) (14) was used when strains were grown with aromatic substrates as sole carbon sources. Glucose (0.5%) and Casamino Acids (0.3%; Difco Laboratories, Detroit, Mich.) were added to VB and BM media as growth substrates when required. *P. aeruginosa* strains were grown at 37°C; *Burkholderia* sp. strain JS150 was grown at 30°C.

Toluene monooxygenase assays. *P. aeruginosa* PAO4032 carrying the plasmids described in Results and Discussion were grown in BM-glucose to the late log phase, harvested by centrifugation, washed twice in 25 ml of 40 mM phosphate buffer (Na_2HPO_4-KH_2PO_4 [pH 7.5]), and then resuspended in 2 to 5 ml of buffer. From this suspension, cells were added to 9.5 ml of phosphate buffer containing toluene (2.8 mM) in 125-ml bottles to yield an A_{425} of 1.0 and a final volume of 10 ml. The bottles were then sealed with rubber stoppers. Cell suspensions were shaken at 25°C, and 0.6-ml samples were collected after 4 and

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20 h. The samples were mixed with 0.6 ml of methanol in 1.5-ml microcentrifuge tubes and then clarified by centrifugation. The supernatants were analyzed by reverse-phase high-performance liquid chromatography (HPLC) to determine the cresol isomer concentration that resulted from toluene oxidation. Product concentration was determined by comparison to a standard curve of chromatogram peak area versus standard cresol concentrations as done previously (19).

Incorporation of ^{18}O into toluene. ^{18}O incorporation experiments were done exactly as described previously (12). Briefly, *P. aeruginosa* PAO4032 carrying the recombinant plasmids noted in Results and Discussion were grown to the late log phase in BM-glucose medium containing toluene to induce monooxygenase activity. The cells were then harvested and washed as described above for the monooxygenase assays; following that, the cells were resuspended in 150 ml of phosphate buffer (final A_{425}, 1.0) and transferred to a three-neck distillation flask. The vessel was sealed with stopcocks with screw-on caps and Teflon-lined septa. Throughout the experiment, the cell suspension was mixed with a magnetic stirrer. Ambient air was removed from the flask by repeatedly evacuating the headspace and replacing the volume with nitrogen. Following the third evacuation, the vessel was partially filled with nitrogen and then brought to ambient pressure with pure oxygen enriched with $^{18}O_2$ (95% atom ^{18}O). The cell suspension was stirred to allow equilibration of the medium, and toluene was then added to the vessel (final concentration, 2.8 mM). This suspension was incubated for 3 h with stirring. Following incubation, the toluene metabolites were extracted from the medium as described before (12) and then analyzed by reverse-phase HPLC and gas chromatography-mass spectroscopy (GC-MS).

Molecular genetic techniques. Total genomic DNA used in initial cloning trials was isolated from strain JS150 by a modification of the Marmur method (15) as described by Scordilis et al. (22). Chromosomal and plasmid DNAs used in Southern hybridization experiments were isolated from strain JS150 by previously described methods (6, 18) and then purified by cesium chloride-ethidium bromide density gradients. Southern hybridization analyses were done exactly as described previously (12); restriction endonuclease-cleaved chromosomal and plasmid DNAs were separated by agarose gel electrophoresis and then transferred to nitrocellulose membranes as described by Chomczynski (1). Other molecular genetic techniques were done by use of standard procedures (18, 21) and as recommended by the reagent manufacturers.

Analytical methods. HPLC separation of metabolic products was done with a system of components from the Shimadzu Corporation (Kyoto, Japan). Mobile-phase flow was controlled by a dual-pump (model 6CL-6B) solvent delivery system. Samples were loaded from either a manual injection port (Rheodyne, Contati, Calif.) or via an SIL-9A autoinjector. Analytes were detected upon passage through a UV-Vis spectrophotometric detector (model SPD-6AV). The detector was coupled to a model CR501 Chromatopac for quantification of analytes.

Analysis by reverse-phase chromatography was done with a 4.6- by 250-mm cartridge column packed with Spherisorb ODSD (5-μm particle diameter; PhaseSep, Norwalk, Conn.). The cartridge was connected to a 17-mm reverse-phase guard column. The mobile phase used in these separations was a mixture of methanol and water (50:50) at a flow rate of 1.5 ml/min. Normal-phase chromatography was done with a 4.6- by 250-mm cartridge column packed with S5 nitrile (5-μm particle diameter; PhaseSep) and connected to a 17-mm normal-phase guard cartridge. The mobile phase consisted of hexane-dichloromethane (85:15) at a flow rate of 1 ml/min.

GC-MS analysis was completed with a Hewlett-Packard (Palo Alto, Calif.) model 5890 integrated GC-MS. Analytes were separated with a 30-m by 0.25-mm capillary column coated with Durabond (DB) 5% phenyl-methylpolysiloxane (J and W Scientific, Folsom, Calif.). Helium was used as the carrier gas. Following sample injection, the oven temperature was held at 35°C for 4 min, increased 5°C/min to 150°C, and held at 150°C for 20 min. The GC was coupled to a Finigan-MAT (San Jose, Calif.) quadrapole MS with an INCOS data system for analysis of the mass spectra that were obtained.

Chemicals. The aromatic hydrocarbons used in this study were obtained from Aldrich Chemical Company (Milwaukee, Wis.). Bacteriological medium components were purchased from Difco. Trimethoprim and ticarcillin were obtained from Sigma Chemical Co. (St. Louis, Mo.). Disodium carbenicillin was purchased from Pfizer, Inc. (Cherry Hill, N.J.). Oxygen enriched with $^{18}O_2$ was from the Mound Isotope Division of the Monsanto Research Corporation (Miamisburg, Ohio). Enzymes and reagents used for DNA manipulations were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and Gibco-BRL, Inc. (Gaithersburg, Md.).

RESULTS AND DISCUSSION

Identification and genetic mapping of the Tb4m from *Burkholderia* sp. strain JS150. When *P. aeruginosa* PAO1c (pRO2016) was grown in BM containing toluene plus glucose as the growth substrate, polar metabolites resulting from toluene oxidation accumulated in the medium. The identity of these products was determined with reverse- and normal-phase HPLC by comparing the column retention times of the unknown products with those of authentic standards. From those analyses, we found that the products included a mixture of cresol (methylphenol) isomers. The predominant isomer was *o*-cresol (12); however, a significant concentration of *p*-cresol was also detected. This finding suggested that the DNA insert contained on pRO2016 included genes encoding toluene-4-monooxygenase activity. To identify the region of DNA that encoded the 4-monooxygenase activity, subclones and deletion derivatives of pRO2016 were made and transferred to *P. aeruginosa* PAO4032, and the constructed strains were tested for toluene-4-monooxygenase activity as described in Materials and Methods. The results of these experiments are summarized in Fig. 1.

Initially, the plasmid construct designated pRO2025 was obtained by deleting the region of DNA sequence mapping from the cloning vector *Cla*I (14.0 kb) recognition site to the *Cla*I site at 8.9 kb in the insert DNA (Fig. 1). *P. aeruginosa* PAO4032(pRO2025) exhibited levels of 4-monooxygenase activity comparable to those obtained from *P. aeruginosa* PAO4032(pRO2016). A second subclone of this region, pRO2038, was constructed by subcloning the internal *Eco*RV-*Cla*I restriction fragment (map coordinates, 3.7 to 8.9 kb) into vector pRO1614. Toluene monooxygenase assays with strain *P. aeruginosa* PAO4032(pRO2038) provided detectable levels of toluene-4-monooxygenase activity following extended incubation periods (20 h), although no product was found after the initial assay period (Fig. 1). The results suggest that the structural genes encoding toluene-4-monooxygenase were confined within the 5.2-kb *Eco*RV-*Cla*I fragment; as a result, the region was designated the Tb4m locus as shown in Fig. 1.

Like Tb2m activity (12), toluene-4-monooxygenase activity was inducible. When *P. aeruginosa* PAO4032(pRO2016) was grown in minimal glucose medium without an aromatic effector present, the strain provided only a low level of 4-monooxygenase activity (1.2 nmol of *p*-cresol/h) compared to that provided by toluene-induced cells (Fig. 1). This finding suggested that regulatory features controlling Tb4m expression were included on the pRO2016 DNA insert. We tested whether the previously identified regulator, TbmR, controlled expression of the Tb4m. For this, we transferred pRO2369 (12), a derivative of cloning vector pRO2321 which contains the *tbmR* locus, to *P. aeruginosa* PAO4032(pRO2038) and determined the level of toluene-4-monooxygenase activity for the resulting strain. We found that the strain, which carried the Tb4m structural genes together with the *tbmR* locus in *trans*, showed induced toluene-4-monooxygenase activity following toluene exposure (Fig. 1). This result showed that the *tbmR* gene product regulated Tb4m as had been observed previously for Tb2m.

^{18}O incorporation experiments—*P. aeruginosa* PAO4032 (pRO2016). Experiments were done to demonstrate that *p*-cresol formation from toluene was due to incorporation of a single atom of dioxygen into the aromatic benzene nucleus. Toluene was incubated with whole cells of *P. aeruginosa* PAO4032(pRO2016) in air or an $^{18}O_2$-enriched atmosphere, and the products resulting for toluene oxidation were isolated and analyzed by GC-MS. The total ion current chromatograms from GC separation of products showed a compound (retention time, 17.25 min) that provided mass spectra characteristic of *p*-cresol. The spectra showed a molecular ion and reference peak of 108 and 107 atomic mass units, respectively, and a fragmentation pattern corresponding well to an authentic *p*-cresol standard. The molecular ion value of *p*-cresol obtained in the $^{18}O_2$-enriched atmosphere increased 2 atomic mass units over the product obtained in air. This shift showed that the oxygen in *p*-cresol was derived from molecular oxygen and that one atom of oxygen was incorporated into toluene to form the

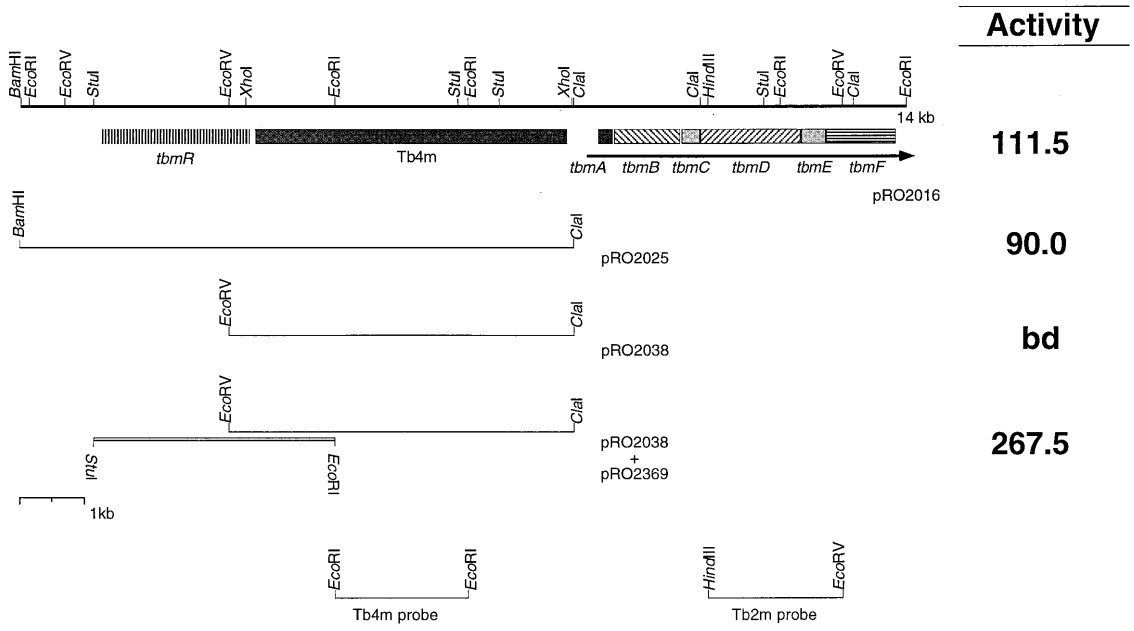

FIG. 1. Physical and genetic map of the 14-kb DNA insert on pRO2016 and the restriction fragments subcloned to localize the region containing the Tb4m locus. The numbers in the column on the right correspond to the toluene-4-monooxygenase activity (in nanomoles per hour) that was measured at the 4-h time point. Reported values are the means of four or more independent trials. The rectangles below the physical map mark the boundaries of the loci and genes for the toluene monooxygenases and regulatory protein. Restriction fragments in the lower portion of the figure correspond to probes used in Southern hybridization analyses (see description in the text). bd, below detection (<0.25 nmol h^{-1}).

product. Accordingly, these experiments confirmed that the oxidation of toluene to *p*-cresol was carried out through an authentic 4-monooxygenase reaction (7).

Phenol hydroxylation by Tb2m and Tb4m. Our previous work showed that the Tb2m encoded by genes on pRO2016 catalyzes the transformation of *o*-cresol to 3-methylcatechol (12). We were interested, first, in determining whether the Tb4m also exhibited cresol hydroxylase activity and, second, if the monooxygenases showed a preference for any of the three cresol isomers. To answer these questions, we determined the level of cresol hydroxylase activity for strains carrying plasmids with the individual monooxygenase and regulatory genes. The experiments were done like the toluene monooxygenase assays except that cresol isomers were substituted for toluene as the test substrates. The results are summarized in Table 1 and include *P. aeruginosa* PAO4032(pRO2016) for comparison. The plasmid designated pRO2037 contains the structural genes for the Tb2m; pRO2037 is a derivative of pRO2016 (Fig. 1), which was deleted for the insert DNA lying between the *Bam*HI and *Xho*I restriction sites at map coordinates 0 to 8.9 kb (12).

The experiments confirmed that the Tb2m was responsible for transformation of the phenolic intermediates in the metabolic pathway. The strain with 2-monooxygenase, *P. aeruginosa* PAO4032(pRO2037, pRO2369), provided levels of cresol hydroxylase activity comparable to those of the strain with both monooxygenases, *P. aeruginosa* PAO4032(pRO2016). However, the strain with 4-monooxygenase, *P. aeruginosa* PAO4032(pRO2038, pRO2369), showed insignificant levels of substrate transformation. These findings were consistent with growth characteristics of strains carrying subclones and deletion derivatives of pRO2016. Plasmids which had deleted or interrupted genes for the Tb2m, e.g., pRO2025, did not allow growth of *P. aeruginosa* PAO1c on benzene or phenol. Therefore, the level of phenol hydroxylase activity from the Tb4m is not physiologically relevant and Tb4m serves only to hydroxylate the unactivated benzene nuclei in the degradative pathways. Based on the concentration of product resulting from cresol oxidation, *p*-cresol was the preferred cresol isomer for the 2-monooxygenase. However, all the cresol isomers were accommodated and hydroxylated by the 2-monooxygenase to yield the corresponding methylcatechol isomers (Table 1).

Regulation of toluene monooxygenase activity. To determine the effector range for TbmR-mediated regulation and whether the effector profiles for the individual monooxygenases differed, toluene monooxygenase activities for strains *P. aeruginosa* PAO4032(pRO2037, pRO2369) and *P. aeruginosa* PAO4032

TABLE 1. Cresol hydroxylase activity from recombinant *P. aeruginosa* strains

Strain	Substrate	Activity with resulting products (nmol h^{-1})[a]	
		3-Methylcatechol	4-Methylcatechol
PAO4032(pRO2016)	*o*-Cresol	175.7	
	m-Cresol	83.0	29.2
	p-Cresol		360.4
PAO4032(pRO2037, pRO2369)	*o*-Cresol	335.0	
	m-Cresol	234.2	1.5
	p-Cresol		531.5
PAO4032(pRO2038, pRO2369)	*o*-Cresol	10.5	
	m-Cresol	BD[b]	BD
	p-Cresol		BD

[a] Values measured following initial incubation period (4 h) represent results for mean concentrations of product from two or more independent experiments.
[b] BD, below detection (<0.25 nmol h^{-1}).

TABLE 2. Induction of toluene monooxygenase activity

Strain	Effector[a]	Toluene monooxygenase activity (nmol h^{-1})[b]
PAO4032(pRO2037, pRO2369)[c]	Toluene	108.2
	o-Cresol	93.2
	m-Cresol	51.0
	p-Cresol	111.7
	Benzene	92.7
	Phenol	182.7
	Chlorobenzene	143.0
	None	1.0
PAO4032(pRO2038, pRO2369)[d]	Toluene	267.5
	o-Cresol	143.7
	m-Cresol	81.5
	p-Cresol	7.0
	Benzene	279.2
	Phenol	106.2
	Chlorobenzene	151.0
	None	BD[e]

[a] Concentration of effector substrates, 3 mM in each trial.
[b] Values measured following initial incubation period (4 h) represent results for mean concentrations of product from two or more independent experiments.
[c] Activity was determined based on concentration of *o*-cresol resulting from toluene oxidation.
[d] Activity was determined based on concentration of *p*-cresol resulting from toluene oxidation.
[e] BD, below detection (<0.25 nmol h^{-1}).

(pRO2038, pRO2369) were determined following growth with various aromatic compounds as inducers. The results of these experiments are summarized in Table 2.

Chloro-, methyl-, and unsubstituted benzenes all induced toluene-2- and toluene-4-monooxygenase activity in strains carrying the individual monooxygenase loci (Table 2). These results were consistent with those obtained in experiments testing monooxygenase activity from *P. aeruginosa* PAO4032 (pRO2016), which showed elevated levels of both 2- and 4-monooxygenase after induction with these aromatic compounds (10). Subsequently, various phenolic compounds were tested as effectors for TbmR activation. We found that phenol and *o*-, *m*-, and *p*-cresol all induced toluene monooxygenase activity in the strains containing plasmids with the individual monooxygenase loci. The 2-monooxygenase activity increased after growth with any of the phenolic compounds; however, phenol exposure induced the highest level of 2-monooxygenase activity. The influence of the methyl substituent position was tested with *o*-, *m*-, or *p*-cresol as the effector. The assays showed that *o*- and *p*-cresol induced 2-monooxygenase activity that was approximately two times that found following *m*-cresol induction. The 4-monooxygenase activity was also induced by phenolic compounds, this despite the fact that these compounds were not substrates for the enzyme. In this set of experiments, growth with *o*-cresol, *m*-cresol, and phenol all induced moderate levels of 4-monooxygenase activity. By contrast, *p*-cresol-induced cells provided minimal levels of 4-monooxygenase activity (Table 2).

The effector range demonstrated for TbmR-mediated regulation appears to establish a positive feedback loop to maximize Tb2m and Tb4m expression. Since both the initial substrates and hydroxylated products induce monooxygenase activity, enzyme activity can be maintained under more growth conditions. For example, toluene induction would lead to the synthesis of the monooxygenases, which, in turn, transform toluene to yield cresol isomers, which also serve as inducers to maintain enzyme synthesis. Although TbmR exhibited a broad effector range, there were differences in effector specificity in relation to both the class of benzene ring substituent and positioning of the substituents on the ring. An example of these differences was observed when *p*-cresol was tested as an effector of Tb2m and Tb4m expression. We found that *p*-cresol minimally induced Tb4m activity, yet among the cresol isomers, *p*-cresol induced the highest level of Tb2m activity (Table 2). This relationship suggests that the TbmR-activator complex binding site for the Tb4m locus differs from that for the Tb2m locus. However, it is interesting that the phenolic compounds served as inducers of Tb4m activity although they were not substrates for the enzyme. This incongruity between substrate and effector ranges suggests that the Tb4m operon may have recruited its regulator from the Tb2m pathway to provide the present degradative system.

Cloning and characterization of genes for a second toluene-4-monooxygenase from *Burkholderia* sp. strain JS150. Our previous work showed that additional loci which had homology with the Tb2m are present in the genome of strain JS150 (12) and suggested that genes encoding other toluene monooxygenases may be carried by the strain. To explore this possibility, additional DNA fragments that encoded toluene monooxygenases were cloned from strain JS150. Direct selection was used to isolate strains carrying recombinant plasmids of interest (2, 3). One plasmid, designated pRO2015, harbored a 20-kb DNA fragment cloned into vector pRO1727 that allowed *P. aeruginosa* PAO1c to grow with benzene as a sole carbon source. *P. aeruginosa* PAO1c(pRO2015) could use phenol or benzene but not toluene or chlorobenzene as the sole carbon source. However, when this strain was grown in BM-glucose medium containing toluene or chlorobenzene, metabolic intermediates accumulated in the medium. The identities of the intermediates were determined by using reverse- and normal-phase HPLC to compare the column retention time of the metabolic products with that of authentic standards. The major product from toluene oxidation by *P. aeruginosa*(pRO2015) coeluted with *p*-cresol. A product that coeluted with *o*-cresol was also observed but at a far lower concentration. The difference in concentration suggested that the *o*-cresol resulted from misdirected hydroxylation of the benzene ring and not the presence of a second monooxygenase like that seen for strains carrying pRO2016. Product peaks which corresponded to 3- and 4-methylcatechol were also found in these analyses; 4-methylcatechol was the predominant isomer, the 3-methyl catechol resulted from hydroxylation of the small amount of *o*-cresol. When chlorobenzene was included in the growth medium rather than toluene, the principal metabolites coeluted with 4-chlorophenol and 4-chlorocatechol, suggesting that the regiospecific oxidation of chlorobenzene was analogous to toluene oxidation for this strain.

^{18}O incorporation experiments—*P. aeruginosa* PAO4032 (pRO2015). The monooxygenation of toluene to *p*-cresol by *P. aeruginosa* PAO4032(pRO2015) was characterized as before by demonstrating that ^{18}O was incorporated into the primary metabolic product. Products resulting from toluene oxidation in air or an $^{18}O_2$-enriched atmosphere were compared by use of GC-MS. The total ion current chromatograms from GC separation of the products showed a predominant signal (retention time, 17.25 min) that provided mass spectrum characteristics of *p*-cresol. The molecular ion value of *p*-cresol obtained in the $^{18}O_2$-enriched atmosphere increased 2 atomic mass units over the value obtained in air. This shift showed that the oxygen in *p*-cresol was derived from molecular oxygen and thereby confirmed that the hydroxylation of toluene to *p*-cresol

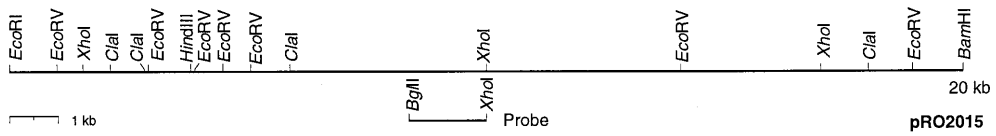

FIG. 2. Restriction map of the 20-kb DNA insert on pRO2015 and the restriction fragment used as a probe in the Southern blot analysis shown in Fig. 3.

by *P. aeruginosa* PAO4032(pRO2015) was carried out through an authentic 4-monooxygenase reaction (7).

Southern hybridization analysis. Comparisons between the DNA inserts cloned in pRO2015 and pRO2016 showed that the DNA fragments were not identical but did have similar regions. Restriction maps of the two inserts were different (Fig. 1 and 2). Southern blotting which used probes derived from the Tb2m (Fig. 1, Tb2m probe) and Tb4m (Fig. 1, Tb4m probe) loci was done to analyze the DNA insert contained on pRO2015 (Southern blot data not shown). The Tb2m probe hybridized with the pRO2015 insert DNA, but blots with the Tb4m probe did not show hybridizing fragments from pRO2015. These results indicated that the gene clusters encoding the toluene-4-monooxygenases contained in pRO2016 and pRO2015 are substantially different although the enzymes carry out similar regiospecific oxidations of toluene and chlorobenzene. The results were also consistent with the suggestion that a single monooxygenase enzyme was encoded by genes contained in pRO2015 to carry out the hydroxylations of toluene and *p*-cresol to yield 4-methylcatechol.

Southern hybridization experiments also showed that the insert DNA on pRO2015 originated from the chromosome of strain JS150. Southern blots hybridized to a probe derived from pRO2015 (Fig. 2) showed a common 8.5-kb *Xho*I restriction fragment in both strain JS150 chromosomal DNA (Fig. 3, lane 1) and in a region internal to pRO2015 insert DNA. Additional DNA fragments that hybridized to the probe were detected in the chromosomal and plasmid lanes. The large fragments in the chromosomal lane could represent an artifact from incomplete DNA cleavage, but the smaller fragment was likely a distinct homologous locus. Similarly, the 6.5-kb band in the JS150 plasmid preparation (Fig. 3, lane 3) did not correspond to the pRO2015 map, indicating the presence of a separate similar region on one of the JS150 plasmids. These loci may represent additional monooxygenase-encoding genes, but this cannot be confirmed since a functional map of pRO2015 was not determined and the gene product from the probe sequence was not defined. However, this region was required for toluene monooxygenase activity. Derivatives of pRO2015 in which the probe region was deleted did not encode monooxygenase activity (data not shown).

Multiplicity of upper pathways for toluene catabolism in *Burkholderia* sp. strain JS150. With the findings described here and previously, three different upper pathways from strain JS150 have been identified for the metabolism of substituted benzenes such as toluene. The individual steps for these pathways are shown in Fig. 4. The end products, 3- and 4-methylcatechol, would be metabolized in the *meta* ring fission pathway. Haigler et al. showed previously that toluene degradation by strain JS150 can be initiated via a dioxygenase reaction to yield toluene-*cis*-dihydrodiol; this intermediate is then transformed by dihydrodiol dehydrogenase to form 3-methylcatechol as shown in Fig. 4A (4). Our work with strain JS150 has shown that the strain also carries genes for toluene monooxygenases. The chromosomally encoded pathway that was cloned on pRO2015 provided one of these monooxygenase-initiated pathways (Fig. 4B). Here, toluene is first hydroxylated to yield *p*-cresol and then is transformed to 4-methylcatechol in a second hydroxylation. The organization of the genes encoding this pathway has not been defined, but preliminary evidence suggests that a single monooxygenase carries out both steps. The toluene-degradative pathway encoded on pRO2016 is better characterized (Fig. 4C). Two regiospecific monooxygenases, Tb2m and Tb4m, initiate the pathway by hydroxylating the aromatic ring at either the 2 or 4 position. The two cresol isomers are then transformed to 3- or 4-methylcatechol by a second hydroxylation step carried out by the Tb2m. Analysis of the nucleotide sequence from the Tb2m locus showed that it is closely related to a group of multicomponent phenol hydroxylases from three *P. putida* strains (16, 17, 24). However, it differs from these enzymes by its ability to hydroxylate unactivated benzene nuclei as well as phenolic compounds (12). The activity demonstrated by the Tb4m was like that reported for the multicomponent toluene-3- and toluene-4-monooxygenases from *Pseudomonas pickettii* PKO1 (19) and *Pseudomonas mendocina* KR1 (26), respectively. Furthermore, a partial nucleotide sequence of the Tb4m locus showed strong genetic similarities among these three monooxygenases (10). Our characterization of the genes contained in pRO2016 showed

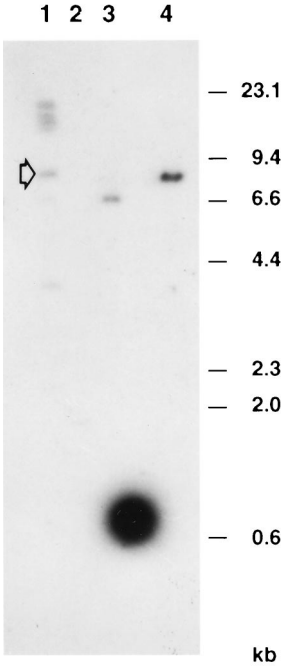

FIG. 3. Autoradiograph of Southern blot showing hybridization of the 1.6-kb *Xho*I-*Bgl*II probe from pRO2015 (Fig. 2) with plasmid and chromosomal DNA preparations from strain JS150 cleaved with restriction enzyme *Xho*I. Lanes: 1, chromosomal DNA from strain JS150 (2 μg); 2, DNA molecular size markers; 3, plasmid DNA from strain JS150 (0.5 μg); 4, pRO2015 cleaved with *Xho*I (0.1 μg). The positions of the molecular size markers are shown on the right. An open arrow indicates the *Xho*I restriction fragment that corresponds to the pRO2015 insert.

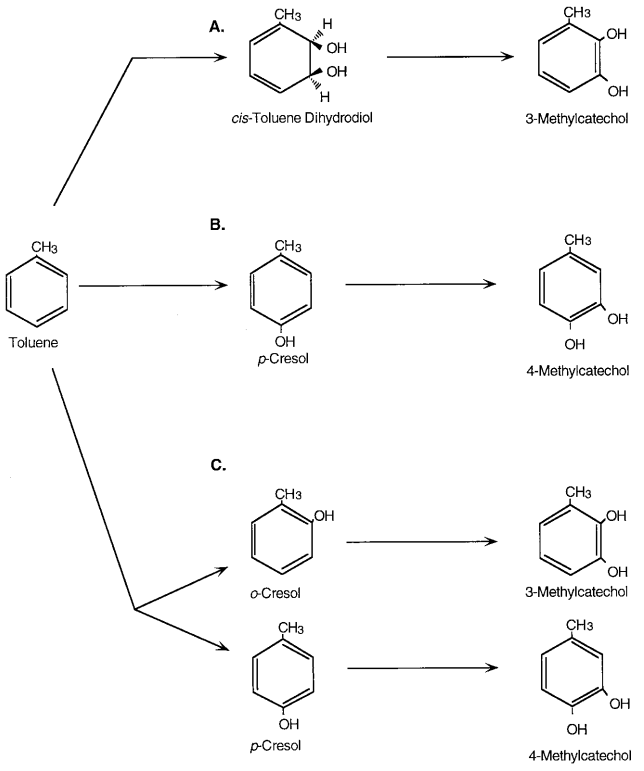

FIG. 4. Proposed pathways for the initial catabolism of toluene by *Burkholderia* sp. strain JS150. (A) Toluene dioxygenase-initiated pathway described by Haigler et al. (4); (B) pathway encoded by genes on pRO2015; (C) pathway encoded by genes on pRO2016.

that two multicomponent monooxygenases were coupled in a single regulon. No previous reports have described a similar biodegradative regulon from other bacterial isolates. This genetic organization may represent a unique biodegradative system for bacterial utilization of aromatic hydrocarbons.

The initial expectation was that our cloning strategy would yield a clone carrying a degradative pathway that could transform unsubstituted, alkyl-substituted, and chloro-substituted benzenes to yield the corresponding catechols. This upper pathway would complement the *ortho*, *meta*-, and modified *ortho* ring cleavage pathways of strain JS150 to allow the previously observed substrate range of this strain (4). Instead, we obtained clones that encoded three different monooxygenases which were distinguishable both genetically and biochemically. Since these monooxygenases are able to hydroxylate some of the same substrates, there appears to be some redundancy in the metabolic makeup of this strain. The reason for this is uncertain, but this capability may promote rapid adaptation to particular selective conditions or environments which give the organism a competitive advantage in nature.

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